PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
C12Q 1/34, 1/28, C12N 9/96
C12Q 1/00

A1

(11) International Publicati n Number: WO 89/ 03888

(43) International Publicati n Date: 5 May 1989 (05.05.89)

(21) International Application Number: PCT/US88/03739

(22) International Filing Date: 24 October 1988 (24.10.88)

(31) Priority Application Number: 116,169

(32) Priority Date: 28 October 1987 (28.10.87)

(33) Priority Country: US

(71) Applicant: GDS TECHNOLOGY, INC. [US/US]; 25235 Leer Drive, Elkhart, IN 46514 (US).

(72) Inventors: FERNANDEZ DE CASTRO, Aurora ; 70421 Hilltop Road, Union, MI 49130 (US). GUPTA, Surendra, Kumar ; 23339 Broadwood Drive, Elkhart, IN 46514 (US). SHANTZ, Steven, Michael ; 719 South 11th Street, Goshen, IN 46526 (US).

(74) Agent: STEPHENSON, Harry, T.; P.O. Box 4659, Elkhart, IN 46514 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), SU.

Published
With international search report.
With amended claims.

(54) Title: TEST COMPOSITION AND METHOD FOR THE DETERMINATION OF ANILIDES

(57) Abstract

A method and unitized test composition is described for the estimation of an anilide in which the enzymatic hydrolysis of the anilide and colorimetric quantitation of aniline or aniline derivative can be done simultaneously. The hydrolysis of the anilide is catalyzed by a known enzyme, arylacylamidase, E.C. 3.5.1.13. Stabilization of the enzyme is provided by the addition of controlled amounts of a compound containing alcoholic and/or aromatic groups such as ortho-cresol, isopropanol or benzoate. Basically, the unitized test composition comprises (i) arylacylamidase, (ii) a controlled amount of an organic compound containing alcoholic and/or aromatic groups which acts as both a stabilizer for the arylacylamidase and forms a colored product with aniline, and (iii) a novel oxidant/catalytic agent for accelerating color developement. In addition, a method for the stabilization of arylacylamidase enzyme is described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BG	Bulgaria
BJ	Benin
BR	Brazil
CF	Central African Republic
CG	Congo
CH	Switzerland
CM	Cameroon
DE	Germany, Federal Republic of
DK	Denmark
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
HU	Hungary
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
RO	Romania
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

TEST COMPOSITION AND METHOD FOR THE DETERMINATION OF ANILIDES

BACKGROUND OF THE INVENTION

This invention relates to a method of stabilizing the enzyme arylacylamidase as well as a simplified enzymatic method and composition for the quantitation of anilide compounds, i.e. N-acylated primary aromatic amines or N-substituted acetamides including N-arylacetamides, such as acetaminophen (4-hydroxy-acetanilide) in samples containing these drugs including biological fluids such as urine, plasma, serum or blood.

Acetaminophen, for example, is commonly used as an analgesic and antipyretic. It is found in many formulations promoted for the relief of pain, cough and colds. Because it can produce adverse side effects, its quantitation or estimation in cases of overdose is particularly important. Cases of overdose may lead to hepatic necrosis with possible fatal hepatic failure as reported in Ann. of Int. Med., 87, 202 (1977). The plasma concentration of acetaminophen is indicative of clinical evidence of liver damage. In cases of overdose known antidotes are administered. Therefore, the simplest and quickest method of testing for this material provides the greatest advantage to the patient.

Several chemical methods for the estimation of an anilide are known. These methods involve the addition of chemical reagents to the solution containing the anilide and the spectrophotometric determination of the resulting colored compound. Examples of these methods

are described by J.H. Routh, et al., Clin. Chem. 14, 882 (1968), S.L. Tompsett, Ann. Clin. Bicchem. 6, 81 (1969), J.P. Glynn, et al. Lancet 1, 1147 (1975) and G.S. Wilkinson, Ann.Clin. Biochem. 13, 435 (1976). In some of these methods, after the anilide is chemically hydrolyzed by acids under a variety of conditions of temperature and time, the resulting aniline or aniline derivative formed is reacted with a substituted phenol or phenolic ether, such as ortho-cresol to give color which can be spectrophotometrically measured at 615 nm.

It has also been known for many years that several organisms produce enzymes (arylacylamide amidohydrolase or arylacylamidase) defined in group E. C. 3.5.1.13, capable of hydrolyzing N-arylacylamides. Examples are R.P. Lanzilotta Ph.D. Thesis, Rutgers University, New Brunswick, New Jersey 1968, N.E. Sharabi et al. App. Microb. 18, 369 (1969), D. J. W. Grant et al., Microbio. 8, 15 (1973). J. Alt et al. in J. of Gen. Microb. 87, 260 (1975) also found another bacterial strain of Pseudomonas (gram negative rods) namely Pseudomonas acidovorans ATCC 15668 which contains an arylamidase E. C. 3.5.1.13 which also hydrolyzes anilides.

Moreover, it has been previously disclosed that the enzymatic hydrolysis of the anilide p-nitroacetanilide to an aniline can be measured spectrophotometrically at 405 nm. The spectrophotometric estimation of the anilide produced by another arylacylamidase enzyme was also reported in U.K. patent GB 2089978 B (1984), U.S. patent 4414327 (1983) and P.M. Hammond, et al. in Anal. Biochem. 143, 152 (1984). In these publications, hydrolysis of anilides is accomplished by an enzyme.

The enzyme E. C. 3.5.1.13 described was derived from a Pseudomonas species, namely Pseudomonas Fluorescens ATCC 39005 and Pseudomonas putida ATCC 39004. The aniline or aniline derivative thus produced was measured spectrophotometrically at 615 nm by a method similar to previously described methodologies using as an oxidizing agent a Cu II salt (or Fe III, chromate, dichromate or permanganate salt), a base in the form of a solution of ammonia and phenol or phenolic ether such as ortho-cresol.

These previous teachings were put to practice by Porton Products in a kit for the determination of acetaminophen in serum which comprises the sequential addition to serum of first an enzyme reagent followed by incubation for three minutes, a second addition of reagent A (1% ortho cresol solution) and a third addition of reagent B (ammoniacal copper solution). The color produced is read spectrophotometrically at 615 nm.

However, there are two problems associated with this methodology, 1) the three step addition of reagents makes the procedure cumbersome if done as a manual method and 2) the method cannot be conveniently automated in most instruments as it would require using three separate reagent channels to do one test plus a preincubation step prior to the addition of the two final reagents.

Accordingly, the present invention provides a simplified methodology for the determination of anilides by providing a) a stabilized arylacylamidase enzyme preparation which can be conveniently integrated

into a stable reagent to be used in any method for the determination of an anilide b) a composition of reagents which can be made into one reagent so that the serum or matrix containing the drug can be added to it and measured spectrophotometrically in a one step reaction c) by providing a format, as one reagent, which can be easily used with automated instruments by occupying only one channel as opposed to three channels and, d) a stable composition of reagents that can be made into a solid-phase reagent test device.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The principle of the method of the present invention is based on the enzymatic conversion of anilide to aniline or aniline derivative by an arylacylamidase enzyme. In the case of acetaminophen, it converts the anilide to 4-hydroxyaniline. The 4-hydroxyaniline can then react with a phenol derivative, such as ortho-cresol, to produce color. This can be shown by the following equations:

$$\text{Acetaminophen} \xrightarrow{\text{Enzyme}} \text{4-hydroxyaniline}$$

$$\text{4-hydroxyaniline} + \text{phenol derivative} \xrightarrow{\text{Catalyst/oxidant}} \text{Colored Product}$$

Because the second react ion described above is relatively slow using the prior art methods, catalyst/oxidants must be used to accelerate the color forming reaction. However, conditions and means previously described in publications to accelerate this reaction tend to inhibit the enzyme reaction. A novel catalyst/oxidant is provided in the present invention to allow all reactions to proceed simultaneously. It has also been unexpectedly found that the present combination of reagents can be accomplished because of the addition or use of a much milder catalyst/oxidant, such as periodate, to the stabilized enzyme and phenolic derivative mixture instead of the ammonium hydroxide or ammoniated copper solution previously disclosed. The addition of such prior art catalyst/oxidants inhibits the enzyme reaction from taking place, thus requiring the previously noted sample/enzyme incubation prior to the addition of reagents. By substituting milder oxidants such as periodates, the enzymatic reaction, as well as the color formation can take place in a one step procedure.

The arylacylamidase enzyme E. C. 3.5.1.13 used in the present invention was obtained from GDS TECHNOLOGY, INC., Elkhart, IN. It was isolated from a Gram positive organism other than Pseudomonas sp. and was obtained in a lyophylized form free from glycerol.

The arylacylamidase enzyme hydrolyzes the amide bond at pH's between 6.5 and 9.5 converting an anilide like acetaminophen to 4-hydroxy-aniline. Preferred buffers are borates and carbonates. It has also been found that The addition of controlled amounts of certain substances containing alcoholic and/or aromatic

groups such as, for example, isopropanol, sodium benzoate and ortho-cresol provide the required stabilization of the enzyme. This stabilization of arylacylamidase is very critical because it allows the preparation of a stable reagent composition in liquid or solid phase format including electrochemical methods which can be used for the determination of anilides.

It is also necessary that a controlled amount of stabilizer be used in the compositions of the present invention. For example it was found that 10-100 mg/100 ml of ortho-cresol enhances the enzyme stability. This is in contrast to previously used higher concentrations of ortho-cresol (about 1%) as described in U.K. Patents 2089978 and 4414327. Such high concentrations tend to inactivate the enzyme and make it unstable.

Importantly and unexpectedly, it has also been found that the same amount of ortho-cresol necessary for the stabilization of the enzyme is sufficient to allow the color reaction to take place simultaneously with the enzymatic reaction. Phenol and other phenol derivatives such as guaiacol can be used to produce color with 4-hydroxyaniline, but at a slower rate. It has been further found that the addition of an oxidizing agent, like periodate, in small amounts considerably speeds up the reaction of the aniline and the phenolic derivative producing color faster. This catalytic agent is also stable at a wide range of pH. Other chemicals like persulfate or hydrogen peroxide and peroxidase also catalyze the color producing reaction.

It has also been found that certain compounds will stabilize the arylacylamidase but do not develop color in the presence of an aniline. Such compounds are sodium benzoate and isopropanol. In such instances color producing compounds such as ortho-cresol or phenol derivatives must be included in the test reagent composition.

EXAMPLES

EXAMPLE 1

Example 1 illustrates the stabilizing effect of some of the above mentioned agents on the enzyme.

Arylacylamidase enzyme was dissolved in a 50 mM Borate buffer at various pH's at widely different concentrations of between 1 and 1500 U/L in the presence of between about 1 and 12 mM ortho-cresol. An equivalent solution of enzyme in buffer was prepared in the absence of ortho-cresol. Both solutions were placed at 37° C for a period of two weeks. The enzyme activity was measured at the beginning as well as at certain times during the two week period. The enzyme activity was determined by a method which uses Tris-HCl pH 8.5 and para-nitroacetanilide as the enzyme substrate. The kinetic assay was performed at 30° C and 405 nm.

Table 1 illustrates this stability of the enzyme in presence of ortho-cresol.

Table 1

REMAINING ENZYME ACTIVITY IN U/ml At 37° C

CONDITION	TIME				
	0	5 hrs	3 days	7 days	14 days
No o-cresol*	13.5	12.9	9.44	4.71	1.87
2.8 mM o-cresol*	13.6	13.5	13.6	12.3	11.8
No o-cresol*	6.26	5.81	4.15	2.01	0.85
4.2 mM o-cresol*	6.25	6.16	6.20	5.66	5.36
No o-cresol**	13.6	5.09	0.06	0.00	0.00
2.2 mM o-cresol**	13.6	13.7	11.2	9.26	5.44
No o-cresol**	6.16	2.27	0.00	0.00	0.00
3.75 mM o-cresol**	6.15	6.20	5.03	4.16	2.36

* pH 8.0

**pH 9.0

At all enzyme concentrations and all pH's, the remaining enzyme activity was much higher when ortho-cresol was present than when it was absent. Higher remaining activity resulted when the test was conducted at room temperature and at 4° C instead of at 37° C. Similar results were obtained when isopropanol and benzoate were added to arylacylamidase enzyme preparations at concentrations of about 0.1 to 12% in the case of isopropanol and of about 1 to 15 mg/ml in the case of benzoate.

Different concentrations of arylacylamidase enzyme were dissolved in buffer at pH's of from 7.0 to 9.5 in the presence of about between 1 to 12 mM ortho-cresol.

This reagent alone produced color with time when serum containing p-hydroxyaniline was added to it. When guaiacol or phenol were substituted for the ortho-cresol, the development of color was even slower. Solutions of an oxidizing agent such as periodate at concentrations of about 1 to 15 mM enhances the speed of the color reaction as did persulfate and a combination of hydrogen peroxide and peroxidase. Periodate was however preferred. Some of the combinations used and the results obtained are shown in examples 2 to 4.

Sodium benzoate was also used to stabilize the enzyme for use in a liquid assay as well as in an electrochemical assay.

Example 2

Arylacylamidase enzyme at a concentrations of 3.5 U/L was dissolved in 25 mM Borate buffer at pH 8.0 containing 3.75 mM ortho-cresol. A solution of 3.75 mM periodate in 50 mM Borate buffer at pH 9.3 was also prepared. The two solutions were mixed at a ratio of 2 parts of enzyme/ortho-cresol solution to 1 part periodate solution to make the final reagent. To 2 ml of the above reagent mixture 50 µl of serum containing various concentrations of acetaminophen was added. The rate of color produced at 37° C was read at 615 nm. Table 2 shows the absorbance rate per minute as a function of concentration.

Table 2

Concentration of p-hydroxyacetanilide in serum in mg/L	OD/min
50	0.0093
100	0.0186
200	0.0411
400	0.0807

The absorbance rate shows a linear relationship with the acetaminophen concentration. Similar but lower readings were obtained when similar concentrations of guiaicol or phenol were substituted for the ortho-cresol.

Instead of periodate, persulfate and hydrogen peroxide and peroxidase were also used to enhance the speed of the color reaction with similar rates of absorbance. The peroxidase system showed catalytic affects at about 0.14 mM H_2O_2 and 0.3 U/ml of peroxidase.

Example 3

Arylacylamidase enzyme at a concentrations of 5 U/L was dissolved in 50 mM Borate buffer at pHs 8.0 and 9.0

containing 4.5 mM ortho-cresol. Solutions of 5 mM periodate in 50 mM Borate buffer at pH's 9.5 and 11.0 were also prepared. The enzyme/ortho-cresol solution and the periodate solution were mixed at a ratio of 2 to 1 respectively. To 2 ml of each the above reagent mixtures 50 µl or 100 µl of serum containing various acetaminophen concentrations was added. The rate of color produced at 37° C was read at 615 nm. Table 3 shows the absorbance rate per minute as a function of concentration in both cases:

Table 3

Concentration of p-hydroxyacetanilide in serum in mg/L	Enzyme sol. at pH 8.0 50 ul in ml/L OD/min	Enzyme sol. at pH 9.0 100 ul serum OD/min
50	0.0111	0.0177
100	0.0221	0.0370
200	0.0422	0.0702
400	0.0807	0.1390

In each case the OD/min shows a linear relationship with the acetaminophen concentrations. Similar but lower readings were obtained when similar concentrations of guiaicol or phenol were substituted for the ortho-cresol.

Example 4

Arylacylamidase enzyme at a concentrations of 3.5 U/L was dissolved in 50 mM carbonate buffer at pH 8.0 containing 3.75 mM ortho-cresol. A solution of 3.75 mM periodate in 50 mM carbonate buffer at pH 9.6 was also prepared. The two solutions were mixed at a ratio of 2 to 1 respectively. To 2 ml of the combined reagent mixture 50 ul of serum containing various acetaminophen concentrations was added. The rate of color produced at 37° C was read at 615 nm. Table 4 shows the absorbance rate per minute as a function of concentration.

Table 4

Concentration of p-hydroxyacetanilide in serum in mg/L	OD/min
50	0.0499
100	0.0885
200	0.1620
400	0.2940

Using carbonate buffer there was also a linear relationship with the rate of color formation and the acetaminophen concentration. Similar but lower readings were obtained when similar concentrations of guiaicol or phenol were substituted for the ortho-cresol.

Example 5

Ten by ten mm squares of filter paper were impregnated with a solution containing arylacylamidase enzyme of various concentrations. For example, 100 U of enzyme per ml of borate buffer, pH 9.0. This solution also contained 10 mM ortho-cresol. The paper was air dried and dipped into a solution containing 15 mM sodium periodate. When 50 ul serum containing different concentrations of acetaminophen was added to these paper strips increasingly deeper shades of blue appeared corresponding to increasing acetaminophen concentrations. The gradation of blue color allowed the estimation of the different acetaminophen concentrations.

Example 6

Different concentrations of peroxidase and hydrogen peroxide were added to 2 ml of 50 mM Borate buffer pH 9.0 containing about 3.5 U arylacylamidase and about 2.5 mM ortho-cresol. Fifty microliters of serum containing different amounts of acetaminophen were then added. The color of the solution produced at 37° C was read at 615 nm. Table 5 shows the rate of color development when 10 U of peroxidase and 50 µl of 0.025% H_2O_2 was added.

Table 5

Concentration of p-hydroxyacetanilide in serum in mg/L	OD/min
50	0.018
100	0.038
200	0.079

WHAT IS CLAIMED IS:

1. A test composition for detecting anilides comprising an arylacylamidase enzyme E.C. 3.5.1.13, an enzyme stabilizing amount of a compound containing alcoholic or aromatic groups which is also capable of developing a colored compound in the presence of an aniline or an aniline derivative and an oxidant/catalyst for accelerating development of said colored compound.

2. A test composition as in claim 1 wherein the compound containing alcohol or aromatic groups is selected from the group consisting of ortho-cresol, phenol and guaiacol.

3. A test composition as in claim 1 wherein the oxidant/catalyst is selected from the group consisting of periodate, persulfate, peroxide and peroxidase compounds.

4. A test composition as in claim 1 which additionally contains a buffer for maintaining the pH of the composition in a range of from about 7.0 to about 9.5.

5. A test composition as in claim 4 wherein the buffer is selected from the group consisting of borates and carbonates.

6. A test composition for detecting anilides comprising an arylacylamidase enzyme E.C. 3.5.1.13, an

enzyme stabilizing amount of a compound containing alcoholic or aromatic groups, an organic compound capable of developing color in the presence of an aniline or an aniline derivative and an oxidant/catalyst for accelerating said color development.

7. A test composition as in claim 6 wherein the compound containing alcoholic or aromatic groups is selected from the group consisting of benzoates and isopropanol.

8. A test composition as in claim 6 wherein the color producing compound is selected from the group consisting of ortho-cresol and phenol derivatives.

9. A test composition as in claim 6 wherein the oxidant/catalyst is selected from the group consisting of periodate, persulfate, peroxide and peroxidase compounds.

10. A unitized test composition for detecting anilides consisting essentially of arylacylamidase enzyme E.C. 3.5.1.13, an enzyme stabilizing amount of ortho-cresol, and a buffer for maintaining the composition at a pH of about 7.0 to about 9.5.

11. A method for the determination of an anilide in an aqueous fluid comprising contacting the fluid with a unitized reagent composition consisting essentially of an arylacylamidase enzyme E.C. 3.5.1.13 and an enzyme stabilizing amount of a compound containing an alcoholic or aromatic groups which also develops color in the presence of aniline or an aniline

derivative, allowing the resulting color to develop and correlating the color developed to the concentration of anilide in the fluid.

12. A method as in claim 11 wherein the reagent composition additionally contains a buffer for maintaining the fluid at a pH of about 7.0 to about 9.5.

13. A method as in claim 11 wherein the compound containing alcoholic or aromatic groups is selected from the group consisting of ortho-cresol, phenol and guaiacol.

14. A method for the determination of an anilide in an aqueous fluid comprising contacting the fluid with a reagent composition consisting of arylacylamidase enzyme E.C. 3.5.1.13, an enzyme stabilizing amount of a compound containing alcoholic or aromatic groups, an organic compound capable of developing color in the presence of an aniline or an aniline derivative and an oxidant/catalyst for accelerating color development, allowing the resulting color to develop and correlating the amount of color developed to the concentration of anilide in the fluid.

15. A method as in claim 14 wherein the reagent composition additionally contains a buffer for maintaining the fluid at a pH of from about 7.0 to about 9.5.

16. A method as in claim 14 wherein the compound containing alcoholic or aromatic groups is selected from the group consisting of benzoates and isopropanol.

17. A method as in claim 14 wherein the color producing compound is selected from the group consisting of ortho-cresol and phenol derivatives.

18. A method as in claim 14 wherein the oxidant/catalyst is selected from the group consisting of periodate, persulfate, peroxide and peroxidase compounds.

19. A test device for detecting anilides comprising a solid state matrix impregnated with the residue of a test composition consisting essentially of arylacylamidase enzyme E.C. 3.5.1.13, and an enzyme stabilizing amount of a compound containing alcoholic or aromatic groups which also develops color in the presence of aniline.

20. A test device as in claim 19 in which the compound containing alcoholic or aromatic groups is selected from the group consisting of ortho-cresol, phenol and guaiacol.

21. A test device as in claim 19 which additionally contains an oxidant/catalyst for accelerating color development.

22. A test device as in claim 21 wherein the oxidant/catalyst is selected from the group consisting

of periodate, persulfate, peroxide and peroxidase compounds.

23. A stabilized reagent composition comprising arylacylamidase enzyme E.C. 3.5.1.13 and a stabilizing amount of a compound selected from the group consisting of ortho-cresol, phenolic derivatives, isopropanol and benzoate compounds.

24. A stabilized reagent composition as in claim 23 wherein the stabilizing compound is ortho-cresol.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/03739

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12Q 1/34, 1/28, C12N 9/96, C12Q 1/00
U.S. C1: 435/18, 435/28, 435/188, 252/89

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/18, 28, 188, 810; 252/89

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstracts Services Online (File CA, 1967-1988).
Automated Patent System (File USPAT. 1975-1988).

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	US, A, 4,414,327 (HAMMOND) 8 November 1983, See Column 6 lines 4-68	1, 2, 4-6, 8, 10-15, 17, 19-21, 24, 3, 7, 9, 16, 18, 22, 23
Y	US, A, 4,668,620 (ARMENTA) 26 May 1987, See Column 1, lines 36-63	3, 9, 18, 22
Y	US, A, 3,557,002 (McCARTY) 15 November 1967, See Column 5, lines 9-25	7, 16, 23

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 December 1988	18 FEB 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Laurie A. Scheiner

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

0 395 680

Application Number

Office

SUPPLEMENTARY EUROPEAN SEARCH REPORT

EP 88 90 9877

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y,D	US-A-4 414 327 (P.M. HAMMOND et al.) * Column 6, lines 4-68 *	1-14	C 12 Q 1/34 C 12 Q 1/28 C 12 N 9/96 C 12 Q 1/00
Y	US-A-4 668 620 (R. ARMENTA et al.) * Column 1, lines 36-63 *	1-14	
Y	US-A-3 557 002 (C.B. McCARTY) * Column 5, lines 9-25 *	1-14	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 12 Q

The supplementary search report has been drawn up for the claims attached hereto.

Place of search	Date of completion of the search	Examiner
THE HAGUE	23-10-1990	HITCHEN C.E.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0401)

CLAIMS:

1. A test composition for detecting anilides comprising an arylacylamidase enzyme E.C. 3.5.1.13, a compound containing aromatic groups which is capable of developing a colored compound in the presence of aniline or an aniline derivative and an oxidant/catalyst selected from periodate, persulfate, peroxide and peroxidase compounds for accelerating development of said color compound.

2. A test composition as claimed in Claim 1 wherein the compound containing aromatic groups is selected from ortho-cresol, phenol and guaiacol.

3. A test composition as claimed in Claim 1 or Claim 2 which additionally contains a buffer for maintaining the pH of the composition in a range of from about 7.0 to 9.5.

4. A test composition as claimed in Claim 3 wherein the buffer is selected from borates and carbonates.

5. A method for the determination of an anilide in an aqueous fluid comprising contacting the fluid with a unitized reagent composition consisting of arylacylamidase enzyme E.C. 3.5.1.13, a compound containing aromatic groups which develops color in the presence of aniline or an aniline derivative and an oxidant/catalyst selected from periodate, persulfate, peroxide and peroxidase compounds, allowing the resulting color to develop and correlating the amount of color developed to the concentration of anilide in the fluid.

6. A method as claimed in Claim 5 wherein the compound containing aromatic groups is selected from ortho-cresol, phenol and quaiacol.

7. A method as claimed in Claim 5 or Claim 6 wherein the reagent composition additionally contains a buffer for maintaining the pH of the fluid in a range of from about 7.0 to 9.5.

8. A method as claimed in Claim 7 wherein the buffer is selected from borates and carbonates.

9. A test device for detecting anilides comprising a solid state matrix impregnated with a test composition consisting of arylacylamidase enzyme E.C. 3.5.1.13, a compound containing aromatic groups which develops color in the presence of aniline and an oxidant/catalyst selected from periodate, persulfate, peroxide and peroxidase compounds.

10. A test device as claimed in Claim 9 wherein the compound containing aromatic groups is selected from ortho-cresol, phenol and quaiacol.

11. A test device as claimed in Claim 9 or Claim 10 wherein the test composition additionally contains a buffer for maintaining the composition at a pH of about from 7.0 to 9.5.

12. A test device as claimed in Claim 11 wherein the buffer is selected from borates and carbonates.

13. A test device as claimed in Claim 11 wherein the matrix is paper.

14. A test composition for the determination of anilides in aqueous fluids comprising, in combination, acrylacylamidase enzyme and a stabilizing amount of a compound selected from sodium benzoate and ortho-cresol.